AZOLE RESISTANCE IN AGRICULTURAL SETTINGS

1. Background and Need. *Aspergillus fumigatus* causes 200,000 life-threatening infections annually and without treatment mortality can approach 90%. *A. fumigatus* can also cause long-term infections in patients with chronic lung conditions including asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD).

Azoles are the major antifungal class used to combat *Aspergillus* species clinically. Azoles are also used to combat fungal pathogens of plants, which are estimated to destroy up to 125 million tons of food crops each year. The environmental use of azoles is thought to be the driving force for much clinical resistance. Azoles inhibit sterol 14a-demethylase, the product of the *cyp51A* gene. Several mutations have been reported in *cyp51A* of azole-resistant *A.fumigatus* from agricultural and clinical settings in Europe; however, many azole-resistant strains do not show any mutations in *cyp51 A.*

There have been recent reports of clinical azole-resistant A. fumigatus strains in the U.S. raising concerns that these major antifungal drugs will become increasingly less effective. Agriculture in the U.S. also depends upon azole fungicides and recent reports suggest that azole resistance is a growing problem in agriculture as well.

2. Project Objective. The overall project objective is to isolate and carefully analyze at least 100 azole-resistant *A. fumigatus* strains from agricultural sites in Georgia and Florida and to use this information to develop a rapid assay for azole-resistant *A. fumigatus* that can be used in the field. The major benefit of the program will be a better understanding of the emergence and potential reservoirs of azole-resistant *A. fumigatus*.

3. Scope of Work. In year 1 we will: a) isolate at least 100 azole-resistant *A. fumigatus* strains from at least 50 agricultural and horticultural sites in Georgia and Florida where fungicide efficacy trials are being conducted or azoles are being used, and b) determine azole-susceptibility, population structure, and presence of mutations known to lead to azole resistance. In year 2 we will: a) investigate azole-resistant strains that lack mutations known to lead to azole resistance, and b) develop a rapid LAMP-based assay for detection of azole-resistant *A. fumigatus* in the field.

4. Technical requirements.

Phase 1: Identify and isolate azole-resistant *A. fumigatus* strains from at least 50 azole-treated agricultural environments. (15% effort)

Task 1: Acquire environmental samples. At least 1,000 soil, cull pile, and compost samples will be collected from at least 50 sites in Georgia and Florida where fungicide efficacy trials using azoles are being conducted or azoles are being applied to control fungal diseases on crop plants, ornamentals in greenhouses and nurseries, and on turf grass.

Deliverable: Report on at least 1,000 agricultural samples from at least 50 Georgia and Florida sites with complete information on location and azole exposure.

Task 2: Isolate azole-resistant *A. fumigatus*. Samples will be dilution plated to medium amended with azole fungicides and resistant *A. fumigatus* will be identified, isolated, and stored for future analysis.

Deliverable: Report on at least 100 azole-resistant *A. fumigatus* strains from 50 agricultural and horticultural sites in Georgia and Florida.

Phase 2: Characterize azole-resistant strains and populations. (30% effort)

Task 3. Azole susceptibility phenotyping. The minimum inhibitory concentration (MIC) values of itraconazole, voriconazole, isavuconazole, and posaconazole for all agricultural strains will be determined using the Etest

system. Isolates showing resistance will be screened again using broth microdilution. Strains with similar azole-susceptibility profiles will be grouped together in later analyses.

Deliverable: Detailed azole-susceptibility profiles of all 2:_ 100 strains in the azole-resistant *A. fumigatus* agricultural site collection.

Task 4. Whole Genome Sequencing (WGS). A subset of at least 100 agricultural samples representing the full range of azole susceptibility and cross-reactivity phenotype classes will be analyzed by whole genome sequencing.

Deliverable: WGS data for at least 100 *A. fumigatus* agricultural strains.

Task 5. Identification of Single nucleotide polymorphisms (SNPs). Single nucleotide polymorphisms (SNPs) will be identified from WGS data and via reduced representation sequencing in agricultural samples.

Deliverable: SNP data for at least 100 *A. fumigatus* agricultural strains.

Task 6. Population structure analysis. SNPs will be used to determine population structure within agricultural *A. fumigatus strains*. Previously developed microsatellite markers will also be used to place agricultural strains within eight recently-identified *A. fumigatus* global genetic clusters. Available SNP data sets from previously characterized clinical strains will also be included in the analysis.

Deliverable: Analysis of population structure of *A. fumigatus* agricultural strains including grouping into global genetic clusters.

Phase 3: Identify the molecular mechanisms conferring azole resistance. (30% effort)

Task 7. *Cyp51A* sequencing. The *Cyp51A* gene along with upstream and downstream regions from each azole-resistant strain and azole-sensitive control strains will be PCR amplified, sequenced, and analyzed for the presence of mutations.

Deliverable: *Cyp51A* sequence data for each strain in the azole-resistant *A. fumigatus* agricultural site collection.

Task 8. *Cyp51A* transcript analysis. *Cyp51A* transcript abundance will be analyzed for representatives of each phenotype class using qRT-PCR.

Deliverable: *Cyp51A* transcript abundance data for each strain in the azole-resistant *A.Fumigatus* agricultural site collection.

Task 9. Transcript analysis of strains showing non-*Cyp51A*-based resistance. For strains that how no explanatory *Cyp51A* mutation(s) or expression level change, RNA will be extracted from mycelia grown with and without azoles. RNAseq data will be analyzed to find common expression patterns, with an emphasis on efflux pumps. A subset of azole-resistant strains with *Cyp51A* mutations representing differing susceptibility patterns will be analyzed in the same way to determine if there are contributions to resistance from other mechanisms.

Deliverables: RNAseq data for azole-resistant *A. fumigatus* agricultural site strains that do not have explanatory Cyp51A mutations.

Task 10. Write and submit paper. Write paper detailing studies with approval/input from CDC mycotic diseases group.

Deliverable: Manuscript submitted to a scientific journal.

Phase 4: Development of LAMP primers and assay for field detection of azole-resistant A. Jumigatus. (25% effort)

Task 11. Develop LAMP primers. Using the sequence information and strains collected from agricultural sites, we will design Loop-Mediated Isothermal Amplification Method (LAMP) primers for the detection of azole-resistant *A. fumigatus*. We will test these primers using representatives from the azole-resistant *A. fumigatus* agricultural site collection and azole-sensitive control strains.

Deliverables: LAMP primers for the detection of *A. fumigatus* and discrimination of azole-sensitive vs. azole-resistant strains in agricultural settings.

Task 12. Develop field assay and test LAMP primers. The LAMP assay will be field tested in selected agricultural settings. Isolate or spore collection methods for the assay will be optimized. Results will be verified in the lab.

Deliverables: Field test data on LAMP assay performance in the field.